

REC'D 03 MAY 2004

WIPO

PCT

Pl 1157287

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

**UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office**

April 28, 2004

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE.**

APPLICATION NUMBER: 60/440,717

FILING DATE: January 17, 2003

RELATED PCT APPLICATION NUMBER: PCT/US04/01162

**By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS**



T. Wallace
T. WALLACE
Certifying Officer

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

01/17/03

JCS9 U.S. PTO

01-21-03 60440717 .011703A/PK

PTO/SB/16 (10-01)

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Approved for use through 10/31/2002. OMB 0651-0032

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. ET 805642048 US

INVENTOR(S)					
Given Name (first and middle (if any))		Family Name or Surname		Residence (City and either State or Foreign Country)	
Rothstein Jeffrey Alicia		Rothstein Ruggerio		Baltimore, M.D Baltimore, MD	
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
GTRAP 3-18: A Regulator of Protein Glycosylation					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input type="checkbox"/> Customer Number _____ OR Type Customer Number here		<input type="checkbox"/> Place Customer Number Bar Code Label here			
<input checked="" type="checkbox"/> Firm or Individual Name		Johns Hopkins University			
Address		111 Market Place			
Address		Suite 906			
City		Baltimore		State	MD
Country		USA		ZIP	21202
		Telephone	410-347-3222	Fax	410-347-3201
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification Number of Pages <u>12</u>		<input type="checkbox"/> CD(s), Number _____			
<input type="checkbox"/> Drawing(s) Number of Sheets _____		<input type="checkbox"/> Other (specify) _____			
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76					
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.		FILING FEE AMOUNT (\$)			
<input type="checkbox"/> A check or money order is enclosed to cover the filing fees		<input type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: _____			
<input checked="" type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.		<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.			
<input type="checkbox"/> The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: <u>NIT R01-N540151</u>					

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME

TELEPHONE

410-347-3222

Date

REGISTRATION NO.

(if appropriate)

Docket Number:

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

CERTIFICATE OF EXPRESS MAILING**EXPRESS MAILING LABEL NO.**

ET 805642048 US

I hereby certify that this correspondence (along with any papers referred to as being attached or enclosed) is being deposited with the United States Postal Service as Express Mail, Post Office to Addressee with sufficient postage in a Flat Rate envelope addressed to Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231 on the date indicated below:

01-17-03
DATE of Signature
And of Mail Deposit

Cheryl Nyquist
Signature

60440717 .011703

U.S. Provisional Patent Application

JHU Ref. No. DM-4165

GTRAP 3-18: A Regulator of Protein Glycosylation

Inventors: Rothstein et al

INVENTION DESCRIPTION

Describe the invention completely, using the outline given below.

1. Abstract of the Invention [Briefly describe the invention]

We have previously described the identification and initial characterization of GTRAP3-18, a novel 22kD C-terminal rEAAT3/EAAC1 associated protein. GTRAP3-18 has a physiologic effect on rEAAT3 activity and decreases its affinity for the substrate glutamate. The mechanism of this effect on transporter activity is linked to the glycosylation state of the transporter. Expression of GTRAP3-18 transporter proteins (e.g. EAAT1, EAAT2, EAAT3, EAAT4) results in decreased glycosylation of the transporter proteins. In parallel, incubation of cell lines expressing these transporters with PNGase F endoglycosidase results in an identical 10 kD decrease in the apparent molecular weight of the transporter protein subtypes. Furthermore, GTRAP3-18 co-expression with transporter subtypes leads to a decrease in sodium-dependent glutamate transport activity, as measured ^3H -L-glutamate uptake. The level of GTRAP3-18 expression determines the relative reduction in rEAAT3 activity. We have expanded our analysis of GTRAP3-18 to include other EAAT subtypes. Expression of GTRAP3-18 is able to modulate both the activity and glycosylation status of other glutamate transporters to an equal extent to its effect on rEAAT3. Therefore, GTRAP 3-18 acts as a general regulator of cytoplasmic glycosylation, and thus is an important tool/therapeutic for protein modulation. GTRAP3-18 could serve as a valuable reagent in the treatment of a large number of CNS and non-CNS disorders that rely on protein glycosylation, including acute neurological disorders such as epilepsy, stroke, traumatic injury, chronic neurological disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, spinocerebellar ataxia, general neuromuscular disorders involving acute and chronic nerve or muscle injury, psychiatric disorders such as schizophrenia, cancer, renal disease.

2. Problem Solved [Describe the problem solved by this invention]

There is a need in the art for compounds that regulate cellular glycosylation, involving membrane proteins such as transporters and receptors, protein that regulate extracellular matrix proteins. This invention identifies a protein, known as GTRAP 3-18, that interacts with glutamate transporter proteins and other proteins to modulate their function thru general regulation of glycosylation. Through their interaction and regulation of glutamate transporter proteins, GTRAP3-18 alters glutamate transport. This provides an important means to regulate synaptic transmission, and is thereby useful for a wide range of neurological and psychiatric conditions.

3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art]

Little is known about the regulation of glutamate transporters and cytoplasmic glycosylation. Regulation could occur at the cell membrane or by signaling to the cell-following binding of glutamate, or through intracellular trafficking cascades. GTRAP3-18 provides one of the first molecules that can regulate cellular glycosylation- relevant to the modulation of a wide range of neural and non-neural proteins.

4. Detailed Description of the invention:

On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

- 1- data pertaining to the invention;
- 2- drawings or photographs illustrating the invention;
- 3- structural formulae if a chemical;
- 4- procedural steps if a process
- 5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.

We have attached a text document.

5. **Workable Extent/Scope** [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a *compound*, describe substitutions, breadth of substituents, derivatives, salts etc., if *DNA or other biological material*, describe modifications that are expected to be operable, if a *machine or device*, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

GTRAP3-18 can regulate glycosylation of cytoplasmic and membrane proteins. Regulation of the glycosylation by GTRAP3-18 can be important in a wide range of proteins that serve as surface receptors (acute and chronic neurological disease), inflammation (CNS inflammation, arthritis, neurodegeneration, AIDS, Cancer), surface extra cellular matrix proteins (tumor growth).

6. **References** [Please list the closest and most relevant journal citations, patents, general knowledge or other public information related to the invention]

Lin CLG, Orlov I, Ruggiero AM, Dykes-Höberg M, Lee A, and Rothstein JD. Modulation of the neuronal glutamate transporter EAAC1 by an interacting protein GTRAP3-18. *Nature*, 2001, 410:84-88

Jackson M., Song W., Liu MY, Jin L., Dykes-Hoberg M., Lin CLG, Bowers WJ, Federoff HJ, Sternweis PC, Rothstein JD. Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. *Nature*, 2001, 401:89-93.

☐ No references available at this time.

INTRODUCTION

The Na^+ -dependent glutamate transporter sub-family rapidly reduces glutamate levels around the synaptic cleft and is critical for preserving nervous system function. They are named excitatory amino acid transporters (EAAT). Two of the five gene products are expressed in astrocytes and glial supportive cells in the CNS, GLT-1/EAAT2 and GLAST/EAAT1. Their predominant functions have been postulated to be protection against excitotoxicity, and the recycling of a neuronal transmitter pool of glutamate. The Na^+ -dependent glutamate transporter EAAT4 is found in Purkinje cells of the cerebellum, and EAAT5 is restricted to the retina. The general neuronal transporter is EAAC1/EAAT3, the predominant high affinity Na^+ -dependent glutamate transporter in cortical neurons. This transporter is found in diverse neuronal populations, including the cortex, hippocampus, and cerebellum.

We have described the identification and initial characterization of GTRAP3-18 (Glutamate Transporter Associated Protein of EAAT3), a 22kD protein isolated from a rat brain cDNA library through yeast-two-hybrid screening using the C-terminal portion of rEAAT3/EAAC1. Our initial publication presented evidence that GTRAP3-18 has a physiologic effect on rEAAT3 activity and decreases its affinity for the substrate glutamate as determined by kinetic analysis (Nature 410 (2001) 84). We continue to characterize this protein and here present evidence that the mechanism of this effect on transporter activity is linked to alterations in the glycosylation state of the transporter. The EAAT family contain conserved N-linked glycosylation consensus sequences and are processed in the golgi to have complex N-linked oligosaccharides on the mature EAAT protein. This complex oligosaccharide may be cleaved *in-vitro* through digestion with an endoglycosidase, endoglycosidase F (PNGase F). Incubation of rEAAT3, rEAAT4, rEAAT1, and rEAAT2 transfected HEK 293 cell lysates with PNGase F endoglycosidase results in a decrease in the electro-mobility of these proteins by 10 kD. We observed that following co-expression with GTRAP3-18, the EAAT family members tested have a decreased electro-mobility. This change is reproducible and creates a protein with the same apparent molecular weight obtained following PNGase F digest. The electro-mobility of co-expressed transporters with GTRAP3-18 can not be decreased further through PNGase F digestion. Co-expression of GTRAP3-18 with the EAAT family members significantly decreases their activity as measured by sodium dependent ^3H -L-glutamate uptake. It appears that the decrease in electro-mobility, resulting from the loss of N-linked oligosaccharides, is responsible for the measured decrease in activity. The amino acid sequence used in our yeast-two-hybrid screen corresponds with the final extracellular loop of EAAT. We are defining the required binding sequence for GTRAP3-18 with the EAAT family members within this conserved domain. From immunofluorescence and co-localization data, it appears that GTRAP3-18 is a reticular protein that is interacting with the extracellular loop of the EAAT as they are nascent chains in the endoplasmic reticulum. GTRAP3-18 appears to alter the glycosylation profile of the EAAT through this endoplasmic reticulum association. GTRAP3-18 is a glycosylation regulatory protein.

METHODS

Antibodies.

A synthetic peptide corresponding to the C-terminal region of EAAC1, GLT-1, EAAT4, and N-terminal region of GLAST was used to generate antisera and has been characterized previously [Neuron 13, 713-725]. The rabbit polyclonal anti-GTRAP3-18 antibodies were raised against peptide sequences from the N and C terminus of the protein sequence. The antiserum for each was affinity purified on a column prepared by coupling the BSA-Conjugated peptide to Affi-Gel 15 (Bio-Rad) (Harlow and Lane, 1988). Anti-HA monoclonal antibody was obtained from BAbCo.

Subcloning of EAAC1/rEAAT3 and GTRAP3-18, and transfection of HEK-293T cells

The eukaryotic expression vectors pcDNA3 and pRK5 were used for expression of cDNAs in the mammalian cell line HEK 293T. Full-length EAAC1 cDNA was subcloned into NotI, EcoRI sites of pcDNA3 vector or into the NotI frame of myc-PRK5 to create rEAAT3-myc fusion. GTRAP3-18 was cloned in frame with an HA sequence tag into PRK5 using Sal/Not sites. The EAAT4 cDNA was subcloned into pcDNA3.1/Hygro(+) (Invitrogen) using the EcoR I restriction site. HEK 293T cells were transfected using the FuGene (Boehringer Mannheim) transfection reagent as directed by the manufacturer.

Measurement of Na⁺-dependent glutamate transport activity.

For transfection studies, HEK cells transfected with pcDNA3.1 or PRK5 were grown in a monolayer on 6-well plates in MEM supplemented with 10% fetal bovine serum and L-glutamine. Assays were conducted 48 hours after transfection using the previously described method [J. Neurosci 18, 2475-2485]. Samples were performed in triplicate with a Na⁺ Krebs buffer control for each using 10 μ M cold glutamate and 2 μ Ci ³H glutamate was added unless noted. The obtained values, in CPM or DPM, from the samples were subtracted from the amount of background uptake as measured in the Na⁺ Krebs buffer controls and normalized for the level of total protein in the well, as measured by the Bradford Protein Assay (Pierce).

Surface Labeling through Membrane Impermeant Biotinylation

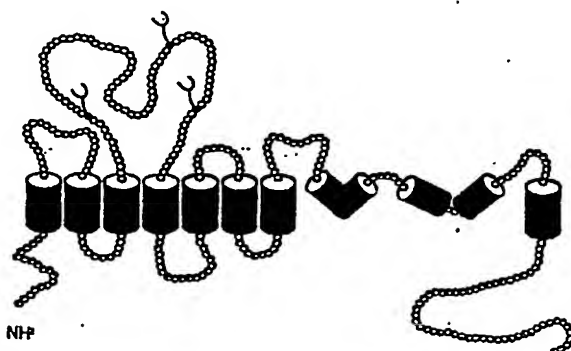
Biotinylation was performed as described with some modifications as in Duan et al [J. Neurosci 19, 10193-10200]. The aliquots of whole cell, intracellular supernatant, and membrane fractions were prepared for Western analysis. SOD1 or actin was used to control for total protein and to determine whether the biotinylation reagent labels proteins in the intracellular compartment. Visualized bands were analyzed using VersaDoc software (Bio-Rad).

Enzymatic De-Glycosylation

Endo H and PNGase were purchased from NEB. Cell lysates following biotinylation were digested at 37°C for 12 hours with gentle shaking with 2U/ μ L of each enzyme in lysis buffer. Lysis buffer consisted of 100 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and protease inhibitor cocktail (Roche) with NP-40 added to 1% preceding PNGase digestion. Non-digested samples were included in the incubation as a control. The biotinylation assay was then completed as described.

Statistics. Statistical differences were determined by Student's *t* test for two-group comparisons.

A)



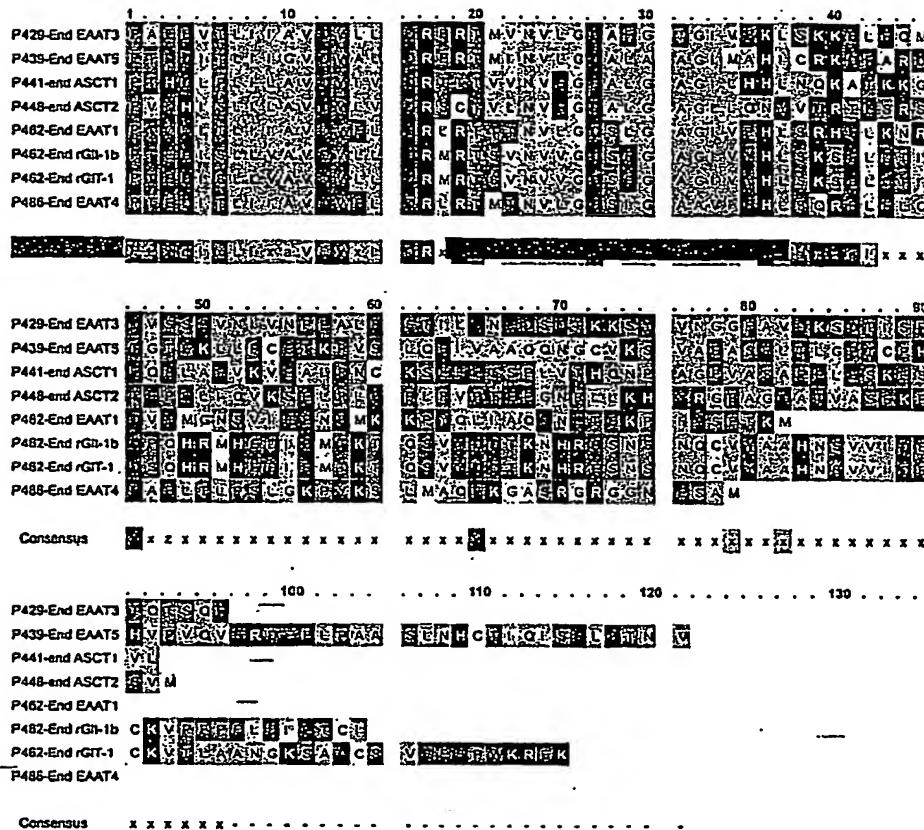


Figure 1. The EAAT (excitatory amino acid transporter) family has a conservation of structure and sequence between the cloned isoforms. Comparison of their carboxy termini reveals divergence following the final transmembrane domain

a) Topology model of rEAAT3 based on solvent accessible amino acid residue studies on EAAT1 published by Seal et al in Neuron Vol 25. Reentrant loop domains are shown in blue.

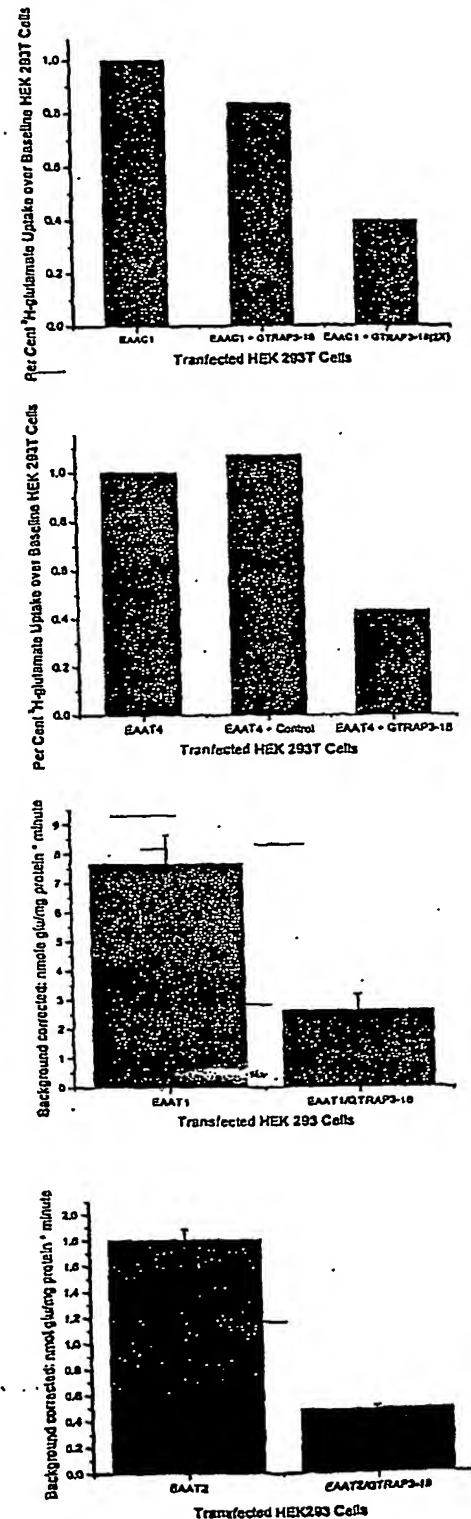
b) Amino acid alignment of C-terminal sequence of EAATs (Excitatory Amino Acid Transporters) and ASCT (Adenine Serine Cysteine Transporter) isoforms. ASCT is the closest related protein family to the EAAT. This alignment highlights the conservation of sequence through the final reentrant (RL3) and extracellular loops and final transmembrane domain (TM8) and the divergence following through the cytoplasmic region. This amino acid sequence for rEAAT3 was used as bait for the yeast-two-hybrid screen that led to the cloning of GTRAP3-18. Preliminary results implicate that the the final extracellular loop may be the interaction site for GTRAP3-18 and the EAAT within the Endoplasmic Reticulum.

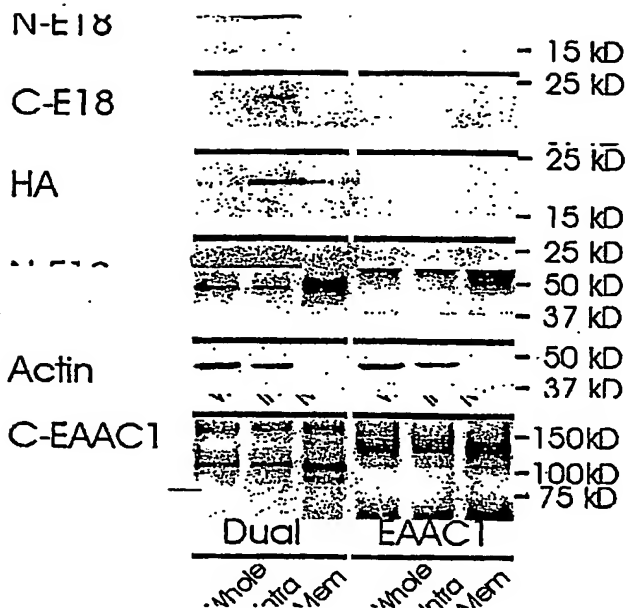
Figure 2. Co-expression of the interacting protein, GTRAP3-18 with the neuronal transporters rEAAT3, rEAAT4, or the astrocytic transporters rEAAT1, rEAAT2 leads to a decrease in Na⁺-dependent

L-[3H]-glutamate uptake in HEK 293T cells.

a & b) Co-transfection of rEAAT3/EAAC1 and GTRAP3-18 in HEK 293T cells at equal transfection ratio reduces EAAC1 Na⁺-dependent L-[3H]-glutamate uptake by approximately 20%, following background correction for the cell line. The decrease in transport can be brought to a higher per cent of control EAAC1 expression by increasing the transfection ratios of EAAC1 to GTRAP3-18 cDNA. Co-transfection of rEAAT4 and GTRAP3-18 had a similar effect on transport

c & d) Expansion of our analysis to the glial transporters rEAAT1 and rEAAT2 also showed a significant decrease in Na⁺-dependent L-[3H]-glutamate uptake following transfection in HEK 293T cells. These transporters have a higher basal uptake activity and were measured at 40 μ M total glutamate. Data are the mean \pm SEM of at least four independent observations and were compared by students *t* test, (** *p* < 0.005), error bars are shown.





60440717.011703

Rothstein and Ruggerio- GTRAP3-18

Figure 3. Co-expression of GTRAP3-18 with rEAAT3/EAAC1 causes a shift in the electro-mobility of rEAAT3 and the other rEAATs.

There is a noticeable change in the expression pattern of EAAC1 following GTRAP3-18 co-expression, seen as a 10 kD decrease in the apparent molecular following co-expression. We used surface protein labeling to determine if this change in the electro-mobility pattern would also affect the cellular distribution of the transporter between the membrane surface and the intracellular trafficking compartment. Biotin was used to label the surface proteins on the HEK 293T-cells and the cell lysates were purified with an avidin conjugated Sepharose column. Three lanes are shown; whole cells, the intracellular portion, and labeled membrane proteins. rEAAT3 is predominantly seen in whole cell and membrane preparations; multiple bands represent dimerization states of the transporter. The final three gels are control for HA-GTRAP3-18 expression.

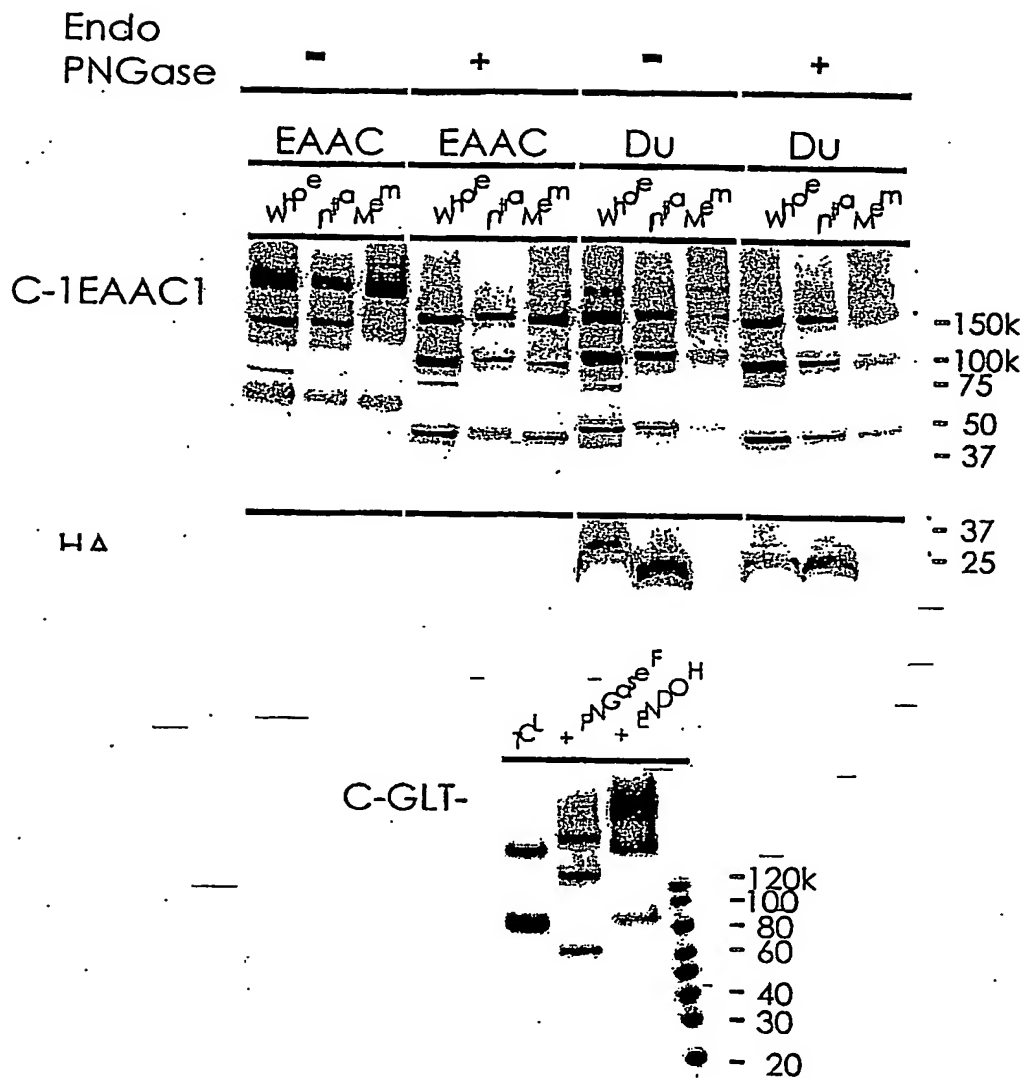


Figure 4. The shift in rEAAT3/EAAC1 electro-mobility following co-expression with GTRAP3-18 can be mimicked by digestion with PNGase F. This result is reproducible for rEAAT1-4. See next column for description.

Figure 4A. The data presented in Figure 3 lead us to the idea that GTRAP3-18 may be altering the glycosylation state of rEAAT3 throughout the cell. This may be the mechanism for the physiological change in activity of the transporters following co-expression seen in Figure 2. To examine this idea, HEK 293T cells were transfected either with EAAC1 or EAAC1 and GTRAP3-18. The cells were harvested after 48 hours and labeled with biotin. The cell lysates were prepared as duplicate samples and incubated for 12 hours with or without the addition of the de-glycosylating enzymes PNGase F and/or Endo H. The supernatant was incubated with immobilized monomeric avidin beads to isolate biotin labeled proteins. Western blots were incubated with C-EAAC1 polyclonal and actin monoclonal antibodies as a marker for intracellular contamination of the membrane fraction. Expression of GTRAP3-18 was visualized with HA monoclonal antibody. Comparison of the effect of digestion with PNGase F and Endo H to the effect of GTRAP3-18 co-expression with EAAC1 indicates that the result is the same reproducible shift in Western immunoreactivity. Digestion of co-expressed lysates did not induce any further change in the apparent molecular weight of EAAC1. This data implicates GTRAP3-18 as a modulator of EAAC1 glycosylation. This experiment was repeated for the other EAAT with the same result (data not shown).

Figure 4B) Transporter N-linked oligosaccharides are processed to completion in the golgi to form complex oligosaccharides. Therefore, they cannot be cleaved with the high mannose specific endoglycosidase H. The molecular weight of PNGase F cleaved transporter is the same as GTRAP3-18 co-expressed transporter.

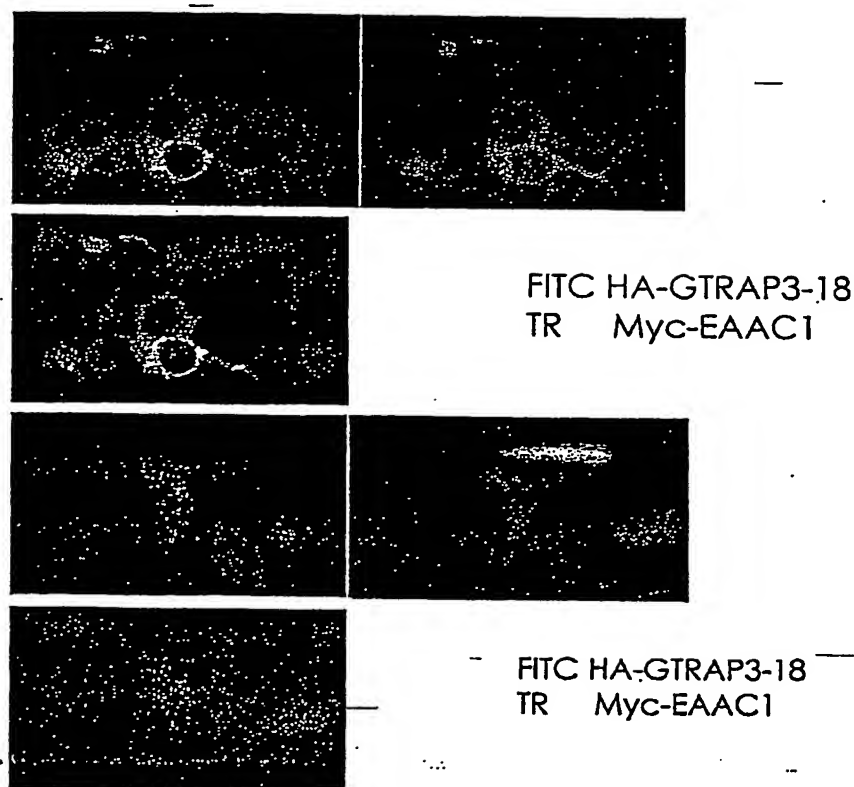


Figure 5. Localization of EAAC1 and GTRAP3-18 in HEK293 cells EAAC1 may be seen both within the cell and expressed as puncta on the membrane surface when labeled by monoclonal myc and anti-mouse Texas Red (Vector). GTRAP3-18 appears as a reticular network throughout the cell and only co-localizes with EAAC1 that is not on the formed into puncta on the cell surface. HA-GTRAP3-18 was labeled by polyclonal HA (BAbCo) and anti-rabbit FITC (Vector). Immunofluorescence photos were taken as a stacked Z-series using a Zeiss LSM 510 confocal.

CONCLUSIONS

- GTRAP3-18 acts to modulate glycosylation of glutamate transporter proteins
- GTRAP3-18 is an interacting protein of the EAAT family discovered by a yeast-two-hybrid screen.
- GTRAP3-18 is able to substantially reduce the activity of co-expressed rEAAT3, rEAAT4, and rEAAT1, rEAAT2 (rEAATs).
- GTRAP3-18 alters the apparent molecular weight of rEAATs following co-expression in HEK 293 cells. This decrease in electro-mobility is the replicated through cleavage of N-linked oligosaccharides using PNGase F.
- GTRAP3-18 appears to be a reticular protein that interacts with

All publications, patents and patent applications disclosed herein are incorporated into this application by reference in their entirety.

GTRAP 3-18 is disclosed in the PCT: WO 01/30968.

For example: "Sambrook et al, Molecular Cloning, A Laboratory Manual (volumes I-III) 1989, Cold Spring Harbor Laboratory Press, USA" and "Harlowe and Lane, Antibodies a Laboratory Manual 1988 and 1998, Cold Spring Harbor Laboratory Press, USA" provide sections describing methodology for antibody generation and purification, diagnostic platforms, cloning procedures, etc. that may be used in the practice of the instant invention.

The following claim(s) of this provisional application are not to be construed as limiting the disclosed invention(s). The claim(s) are included for compliance with patent application structural regulations that may be imposed by international patent offices.

We claim:

1. A method of identifying candidate compounds that modulate cellular glycosylation comprising:
 - introduction of a test compound to GTRAP3-18
 - assaying for binding of the test compound to GTRAP3-18
 - wherein binding of the test compound is correlated to its ability to modulate cellular glycosylation.